



Signal Transduction: Evolution of an Idea

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In general there is no set of observations conceivable which can give enough information about the past of a system to give complete information as to its future.

Norbert Wiener

Think simplicity; then discard it.

Alfred North Whitehead

I was born in 1925, a time when there were no talking movies, radio was just emerging as a popular listening device, newspapers printed important information, and libraries were sources of both pleasure and learning. My father's grocery store (above which we lived) was a community center where people from blocks away would come for their groceries and to gossip. We knew or knew about everyone in our neighborhood. In that atmosphere I grew up as a young man feeling the warmth of this community. Retrospectively, I have come to realize how important this long-gone community and the intense human relationships have been to my development as a scientist. My scientific neighborhood encompasses a place where cultural and language differences have been melded seamlessly and with synergy to promote communication, to expand knowledge with a kinship of purpose, and to create new thought. Nature, which we often equate with our genetic make-up, and Nurture, which symbolizes our environment, interact mutually and synergistically in this community. These are the forces that have given meaning to life; i.e., the parable of which comes first, the chicken or the egg, is not of biological importance.

My lecture symbolizes my interest in societal/cellular relationships and concerns the broad issues of biological communication. The first half deals with the development of the concept of transducers and their role in cell signaling. Since this concept is still at an evolutionary phase, I conclude with a hypothesis which in its simplest message argues that biological communication consists of a complex meshwork of structures in which G-proteins, surface receptors, the extracellular matrix, and the vast cytoskeletal network within cells are joined in a community of effort, for which my life and those of my colleagues is a metaphor.

Receptors, Allostery, and the Second Messenger Theory

The concept of receptors as sensory elements in biology has a long history. Early in this century Paul Ehrlich realized the importance of surface receptors and postulated a "lock-and-key" theory to explain their interactions with antigenic materials and drugs. Today, it is understood that receptors are proteins with the patterns of design and malleability of structure required for discriminating between an extraordinary variety of chemical signals. My interest in receptors began in the early 1960s, when I uncorked the means of freeing adipocytes from their tissue matrix by collagenase treatment and found that insulin at physiological concentrations stimulated glucose uptake (1). Searching for the possible site of action of the hormone, I tested the effects of treating adipocytes with phospholipases and proteases on the assumption that, if the surface or plasma membrane contains insulin receptors, these digestive enzymes might prevent insulin action. Surprisingly, phospholipases mimicked the known actions of insulin on glucose utilization and protein synthesis (2,3). Based on such observations I postulated that insulin might act by stimulating phospholipases (4), which was not a bad hypothesis in view of the accumulated evidence of the importance of phospholipases in mediating the actions of a variety of hormones (5).

During the 1960s, two major theories influenced the course of my research on hormone receptors. One was the "second messenger" theory (6,7). This theory suggested that extracellular or primary messengers in the form of hormones or neurotransmitters act through receptors that regulate the production of 3'5'-adenosine monophosphate (cyclic AMP), considered to be the intracellular messenger that mediates the actions of hormones on all aspects of cellular metabolism, growth, and differentiation. The perceptions of Monod and colleagues that led to their incisive theory of allosteric regulation (8) blended beautifully with Sutherland's theory that receptors are structurally and functionally linked to the regulation of cyclic AMP production. Overwhelmingly persuasive was the notion that adenylyl (now adenylate or adenylyl) cyclase is an

allosterically regulated enzyme system consisting of two distinct sites, receptors and catalytic. Located at the surface or plasma membrane of cells, the asymmetric positioning of these sites—the allosteric hormone-sensing sites on the exterior and ATP-utilizing catalytic sites at the interior surfaces of the membrane—provided a logical framework for investigating the molecular basis for hormone action. My attention shifted from insulin to those hormones known to stimulate the production of cyclic AMP in fat cells.

Multireceptor Adenylate Cyclase System in Adipocytes

At the time, the only specific assay for cyclic AMP production relied on a complicated, time-consuming bioassay. Krishna et al. (9) and later Salomon et al. (10) developed relatively simple chromatographic assays which for the first time allowed rapid, multiple assays of adenylate cyclase. When Lutz Birnbaumer arrived in my laboratory in 1967, that assay literally danced under his extraordinary prowess, yielding information that laid the foundation for the concept of transducers. Before he joined my laboratory, I had developed a rapid method for obtaining fat cell membranes (called "ghosts") responsive not only to insulin but also to various hormones that stimulate cyclic AMP production and resultant lipolysis in fat cells (11). These hormones included epinephrine, adrenocorticotropin hormone (ACTH), thyroid-stimulating hormone, leutinizing hormone, secretin, and glucagon, and fluoride ion. The latter, shown previously to stimulate adenylate cyclase in a variety of cell membranes (6), activated the fat-cell system by a magnesium-dependent process displaying a Hill coefficient of 2.0, suggesting that the system may contain at least two sites of magnesium action, one certainly an Mg-ATP complex at the catalytic site. That a regulatory site for magnesium exists was suggested by the finding that both ACTH and fluoride markedly reduced the concentration of magnesium ions necessary for stimulation of activity (12). The kinetics of ATP action proved too complicated for interpretation at the

This article is dedicated to all my colleagues, former and present, who contributed heavily to the concept of signal transduction. Without their efforts, the field of G-proteins would not have existed.

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time. Not realizing that ATP was contaminated with GTP, we couldn't interpret what later proved to be the stimulatory and inhibitory actions of GTP on adenylate cyclase systems. The lesson is clear to me today: never attempt to interpret a hyperbolic curve; it describes the behavior of the entire universe!

Demonstration of Distinct Hormone Receptors

Much of our energy and time was devoted to delineating the receptors for the hormones that stimulated the cyclase system. The pharmacology of the peptide hormone receptors was essentially unknown and necessitated a variety of indirect tests, including the effects of proteases, inhibitory analogs, and differential ion dependencies, which combined suggested that each of the hormones stimulated cyclase through distinct receptor types. Since the enzyme system and the receptors were contained in the same cell, these findings allowed us to test a fundamental question: do all of the hormones operate on the same enzyme or, as depicted in the Sutherland model, is each hormone receptor coupled to separate cyclase models? The various hormones were tested at maximal and submaximal concentrations alone or combined with the other hormones. Synergy was seen with some combinations, but, most importantly, additivity of response was not obtained with maximal concentrations of the hormones (13). Similar findings were reported simultaneously (14). Although not proof, we argued that it is likely that the fat-cell cyclase system consists of multiple receptors interacting with a common catalytic unit. Conceptually, the picture that emerged is that each receptor contains specific binding regions and some common structural element that interacts with the catalytic component to stimulate conversion of MgATP to cAMP. At that time we considered that the catalytic component contains the regulatory site for magnesium ions and is the site of action of fluoride ion. Lipids were somehow involved in the structural interactions between receptors and catalytic unit because, unlike fluoride action, hormone action was exquisitely sensitive to agents (phospholipases, detergents) that affect membrane structure (15). It was clear that hormone action involved a more complex structural and regulatory enzyme system than originally conceived. It was inconceivable to me that several hormone receptors could be structurally annealed to the same enzyme (I referred to this problem as "too many angels on a pinhead"). A new concept of hormone action had to be considered.

Informational Processing: The Concept of Transduction

At that time my thinking on the subject of how hormonal information is transferred across the cell membrane and translated into action was greatly influenced by the theories of informational processing proposed by Norbert Wiener (16), the originator of cybernetic theory. This subject was introduced to me by Oscar Hechter, who had previously proposed several important theoretical considerations concerning hormone action. Hechter was the first to question the proposition that hormones directly acted on the adenylate cyclase enzyme (17). Through lengthy discussions at a downtown hotel bar in Washington, DC prior to a meeting that I had organized at NIH to honor Sutherland, we arrived at the concept of transduction as a means of coupling information between signal-activated receptor and regulation of adenylate cyclase. Given the paucity of knowledge at that time, the concept of informational processing was put in abstract cybernetic terms: discriminator for receptor, a transducer, and an amplifier representing adenylate cyclase because of the large increase in cyclic AMP generated when converted to its activated state. The transducer is a coupling device designed to allow communication between discriminator and amplifier. At the meeting I presented this idea, illustrated (but without participation of magnesium and GTP at that time) in Figure 1. We considered the possibility that magnesium ions and lipids participated in the transduction process, but we realized that the transducer concept required fleshing out with more evidence on the structure/functional relationships between receptor and enzyme.

Actions of GTP and Glucagon on Liver Cyclase

Because of the experimental complexity of studying the multireceptor adenylate cyclase system in rat adipocytes, my colleagues (Birnbaumer, Pohl, Krans) and I turned our attention to the glucagon-sensitive adenylate cyclase system in liver. To

some extent this change was made because of the historical significance of the hepatic system in hormone action and, coincidentally, because Neville (18) at NIH had reported purification of rat liver plasma membranes by a relatively simple procedure. As importantly, we radiolabeled glucagon with ^{125}I , making it possible to investigate both the nature of the glucagon receptor and the relationship between hormone binding and hormonal activation of adenylate cyclase.

Michiel Krans began the glucagon-binding studies with our findings that hormonal activation of adenylate cyclase in liver membranes rises within seconds and falls rapidly when the hormone is displaced by an antagonist such as des-his-glucagon, which proved later to be a weak, partial agonist. Our expectations were that binding of ^{125}I -glucagon would proceed rapidly (within seconds) and would be reversed easily by washing the membranes free of medium containing the hormone. Instead, Krans observed that binding was extremely slow, requiring at least 20 min before reaching a plateau. Extensive washing under a variety of conditions failed to remove the bound material. None of the binding characteristics fit with the kinetics of hormone action. However, the medium used for binding contained nothing but salt and buffer, whereas the cyclase assay medium contained multiple components including the substrate, MgATP. A dramatic change resulted when all of the cyclase-ingredients were added to the hormone-binding medium. The level of bound hormone at steady-state was drastically reduced; maximal binding was attained within seconds. We subsequently found that ATP was the principal culprit. Realizing from painful experience as a graduate student that commercial preparations of ATP contain a variety of contaminating nucleotides, I tested many types of purine and pyrimidine nucleotides. GTP, GDP, and ITP were the only nucleotides that mimicked the effects of ATP. Most importantly, the guanine nucleotides acted at concentrations much lower (two to three

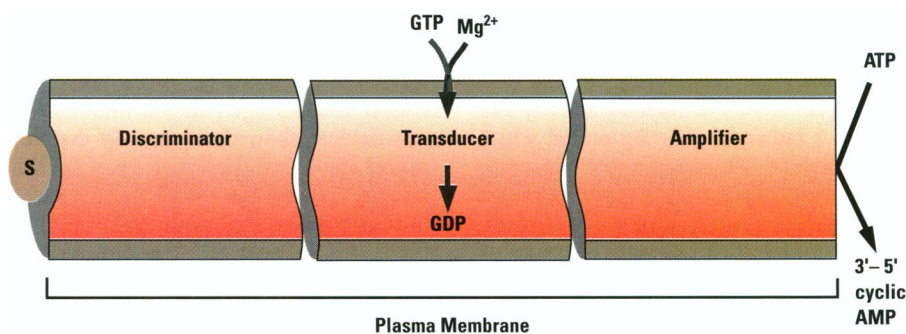


Figure 1. An early version of signal (S) transduction employing abstract terms to describe receptors, GTP-binding proteins, and effectors such as adenylate cyclase.

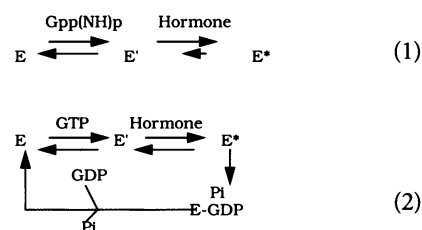
orders of magnitude) than ATP. GppCp, a poorly hydrolyzable analog, also acted, although its effects required much higher concentrations compared to GTP or GDP. Each of the nucleotides induced rapid release of prebound glucagon from its receptor. We established that guanine nucleotides act by lowering the affinity of receptor for the hormone (19).

At that point the central question was the possible relationship of this effect of GTP on hormone binding to the actions of glucagon on adenylate cyclase activity. To avoid the problem of contaminating GTP in the assay for the enzyme, we prepared ^{32}P -App(NH)p as substrate using a biosynthetic method. This analog proved stable to degradation by ATPases in the membrane. Under these conditions, glucagon did not stimulate adenylate cyclase unless GTP was present in approximately the same concentrations that affected the affinity of the receptor (20). Subsequently, Lin and Salomon (21) demonstrated that hormone and GTP concertedly and rapidly induced the active form of the enzyme. Glucagon, moreover, reduced the small lag in activation given by activating nucleotide alone. The die was cast; logically GTP acts at the transduction process along with magnesium ions (Fig. 1). Although the components of the informational processing system remained unknown, there was little doubt in our minds that a transducer exists and that this crucial component mediates the transfer of information between receptor and enzyme.

GTP Hydrolysis

Because GTP was susceptible to hydrolysis by nucleotidases in membranes, our next objective was to substitute GTP with a nonhydrolyzable derivative. Taking a cue from our experience with App(NH)p, Gpp(NH)p was synthesized. A few months later, we found that Gpp(NH)p caused the enzyme's activity to "take off" to an extent not even seen with fluoride ion. To our amazement, the normally unstable cyclase system remained fully active even after 3 days at room temperature. We then tested Gpp(NH)p on a variety of cyclase systems using every cell membrane preparation we could obtain. All showed the same phenomenon (22). Gpp(NH)p, unlike hormone plus GTP, stimulated activity following a rather lengthy lag period which was shortened considerably when hormone was added (21). Salomon investigated the binding of ^{32}P -Gpp(NH)p to liver membranes and found substantial guanine nucleotide-specific binding, far in excess of the number of glucagon receptors (23). These findings were discounted by others because of the seeming disparity in the levels of glucagon receptor and guanine

nucleotide binding sites. However, it was not understood at the time that multiple types of receptors interact with several types of GTP-binding proteins; that story evolved nearly 10 years later. The key elements of signal transduction gained from these findings were that Gpp(NH)p binds to the liver membranes in the absence of hormone, whereas glucagon quickened the ability of the nucleotide to activate adenylate cyclase, not vice versa. These findings, plus modeling of the kinetics of Gpp(NH)p/Mg (24), gave rise to a three-state model (Eq. 2) in which hormones act by promoting the conversion of the nucleotide-bound E' state to the activated state (E*).



However, with 21 parameters using just Mg^{2+} and Gpp(NH)p concentrations as variables, we realized that this model yielded only an approximation of what must be a very complicated system.

At about the same time, Schramm, in a series of beautifully executed experiments, demonstrated that Gpp(NH)p acted in a pseudo-irreversible fashion; i.e., removal of the nucleotide from the medium after incubation resulted in retention of the high level of cyclase activity (25). Based on this finding with Gpp(NH)p taken together with the inability of GTP alone to stimulate activity, we proposed that the transducer must have the capacity to hydrolyze GTP. When GTP was substituted for Gpp(NH)p in the modeling of the liver system's kinetics (Eq. 2), the data fit with the activated state (E*) being the state in which GTP was converted to GDP + P_i. In this fashion, it could be understood why activation by GTP and hormone involved essentially no lag period, whereas with Gpp(NH)p + hormone, the lag was shortened but persisted. GTP turnover, in this model, is required for the rapid, reversible actions of the hormone. A few years later, Cassel and Selinger, in a brilliant set of experiments, showed conclusively that hormones stimulated the hydrolysis of GTP to GDP + P_i. From these findings, they elaborated the theory that hydrolysis of GTP to GDP is the "turn-off" reaction and the resultant bound GDP converts the transducer to its inhibitory state (26). Hormones promote the displacement of GDP and its exchange with GTP; this exchange reaction is the key to hormonal

activation of G-proteins. Nucleotide exchange and GTP-hydrolysis are fundamental to the regulation of all types of G-proteins that have been examined to date. Not considered in this theory, however, is that the overall turnover of GTP is a complex set of reactions including hydrolysis and subsequent release of phosphate from a bound state. In a detailed study of the light-activated rhodopsin system (27), it was suggested that hydrolysis of GTP is a very rapid process, whereas the rate-limiting step is the release of inorganic phosphate from its binding sites on transducin, the G-protein responsible for activation of phosphodiesterase in rod outer segments. This proposal fits with the prolonged activation by fluoride (complexed with aluminum or magnesium ions), which most likely acts by binding to the same magnesium-phosphate binding sites on transducin.

Dual Stimulatory and Inhibitory Actions of GTP and Fluoride

The multireceptor fat-cell system proved invaluable not only for investigating the multiple actions of hormones. It provided the first insight that adenylate cyclase is both inhibited and stimulated by two independent processes involving GTP and fluoride. Löw and Harwood found that fluoride ion and both GTP and Gpp(NH)p induced stimulation and inhibition of the enzyme as the concentrations of these agents were increased (28,29). The mechanism was elusive until Hirohei Yamamura (30) noted marked differences in the properties of the stimulatory and inhibitory phases. Subsequent characterization of the dual process (31) and the discovery (32) that the fat cell contained adenosine receptors that induce inhibition of adenylate cyclase via a GTP-dependent process finally placed the inhibitory role of guanine nucleotides on the same level of importance as the stimulatory process. From these studies arose the new concept of dual regulation of adenylate cyclase by hormones, guanine nucleotides, and fluoride ion (33). Implicit in the argument was the understanding that transduction involving stimulation and inhibition must be exercised through distinct GTP-binding proteins. We called them "nucleotide regulatory proteins" (abbreviated N) because ITP was also active. Thus arose the nomenclature N_s and N_i, now popularly known as G_s and G_i.

One logical consequence of these findings is that G-proteins are independent of both receptors and adenylate cyclase. Pfeuffer's purification of a 42 kDa protein that he could label by incubating membranes with ^{32}P -NAD and cholera toxin (34,35) provided the first tangible evidence for the existence of G_s, the cyclase stimulatory transducer. It had been earlier discov-

ered that cholera toxin greatly increased the production of cAMP in intestinal cells, suggesting that the toxin acts on the adenylate cyclase system [reviewed by Gill (36)]. Later, pertussis toxin (37) provided the means for detecting and purifying G_i and G_o . Meanwhile, in the laboratory of Tompkins, it was found that treatment of cultured lymphoma cells (rat S49) with cyclic AMP resulted in cell death (38). Based on this phenomenon, Tompkins and co-workers isolated surviving mutant forms, one of which was eventually shown to lack the ability of Gpp(NH)p and fluoride ion to stimulate the enzyme; epinephrine action was also abolished (39). Using the mutant called AC⁻ (because it was mistakenly thought to lack adenylate cyclase), Gilman and his colleagues (40,41) subsequently demonstrated that supplementation with extracts from wild-type cells restored both hormonal action in a GTP-dependent fashion and the actions of Gpp(NH)p and fluoride. This assay proved useful for the first purification of what was then called G/F factor, now known as $G\alpha_s$, the transduction protein(s) responsible for stimulating adenylate cyclase.

During this period, studies in our lab (42,43) showed that hormone receptors linked with G_s displayed very different physical and kinetic properties from those observed when adenylate cyclase was linked (after activation) with G_s , suggesting either different states or different forms of the GTP-regulatory process. Finally, and perhaps most critically, was the discovery by Bitensky and colleagues (44) that light-activation of a cyclic GMP phosphodiesterase in rod outer segments was mediated by a guanine nucleotide-dependent process, similar to the actions of guanine nucleotides on adenylate cyclase. By 1980 it was clear that the actions of guanine nucleotides were not confined to the adenylate cyclase system. In a brief overview (33), I proposed that there must be several types of GTP-binding proteins which I called N_s , N_i , N_t (now transducin), and N_x , that mediate the actions of hormones on a number of effector systems. N_x was postulated when I learned that GTP affected the binding of agonists to receptors known to alter calcium uptake in liver cells (45). By 1990, those predictions were proven correct. However, the number and variety of GTP-binding proteins involved in signal transduction are now greater than I had imagined.

General Characteristics of Guanine Nucleotide Action

Within the decade of the 1970s, some of the fundamental characteristics of receptor systems coupled through GTP-binding proteins had been delineated. What followed in the ensuing 20 years was the elaboration of

the types of G-proteins, now about 20. Beginning with transducin (46), it emerged that G-proteins are constructed of three types of subunits, an α -subunit uniquely capable of binding and degrading GTP and a tightly knit complex of β and γ subunits. This discovery, eventually established for all G-proteins coupled to receptors (47), opened up a new chapter in signal transduction which, in recent years, has helped to explain the pleiotropic actions of hormones.

Topological Disposition of Components

One of the most difficult problems in membrane biology is to understand how the membrane's components are organized or structured within the plane of the membrane. The topological relationship of membrane proteins to the exterior and interior components of the cell presents another major problem. The "mobile receptor" concept introduced the notion that receptor proteins are free to move rapidly within the membrane. In the case of receptors linked to G-proteins, this concept gave rise to the hypothesis that hormones act by stimulating the engagement between receptors and G-proteins. The "collision-coupling" model (48) attributes the rate of cyclase activation to the frequency and efficiency of collisions between agonist-bound receptors and G-protein; in this manner any one receptor can activate a number of G-proteins due to the free mobility of each component. The rate of activation of G-proteins (and adenylate cyclase) are directly proportional to the number of agonist-occupied receptors.

Although kinetic analysis can provide important insights into mechanism, in reality the fundamental question is how the different components are constructed and distributed in the plane of the membrane so that they interact with the observed efficiency and rapidity. The logistics of the encounters are obviously better if the membrane is packed with receptors, as in the case of rhodopsin in rods or cones which is in large excess of G-proteins and effectors. However, in most cells hormone receptors are present at relatively low concentrations.

For this reason, I have thought that receptors and G-proteins may be precoupled and that hormones act by altering the nature of the coupling process. This notion now seems justified based on biophysical studies which reveal that receptors are complexed with G-proteins and that such complexes are confined within matrixlike, specialized domains (49). In fact, receptor-coupled signaling processes in general now seem more Bhudda-like in their structures, both in their stationary setting and the multicomponent structures which appear to interact in a flickering fashion, more in

keeping with the ephemeral relationship between action and inaction, between life and death.

The major concern in my laboratory starting in the late 1970s was the structure of the hormone-sensitive cyclase systems as they exist in their native membrane environment. I had learned of target or irradiation analysis from a report that target analysis might be useful for discerning the nature of the interactions between the components of the glucagon-sensitive system in liver membranes (50). The interpretations of the data were based on the mobile receptor theory. Of major concern to us was the fact that irradiation studies were carried out with freeze-dried material. We had learned that freeze-drying of liver membranes, for example, led to drastic reductions in hormonal regulation of adenylate cyclase. We decided to use this technique employing a different protocol not involving freeze-drying.

Fortunately, on the floor above my lab dwelled a scientist with the necessary credentials. Ellis Kempner had conducted his graduate thesis on the usage of irradiation analysis, knew both its promises and its faults, and became interested in our problem. As importantly, Werner Schlegel, a young scientist from Switzerland trained in biophysics, had just arrived in the lab looking for a suitable research problem. Schlegel and Kempner began a project which became the focal point of our research for the past 15 years.

Target Analysis

Schlegel and Kempner ultimately worked out procedures that fully preserved activity and, indeed, provided the first detailed *functional structure* of each component of the glucagon-sensitive system in liver membranes and the hormone-sensitive, stimulatory and inhibitory structures in rat adipocytes (51,52). I emphasize the phrase "functional structure" since the analysis measures the exponential decay in activity in relation to the energy input of electrons that bombard the system; this relationship provides the functional mass. As reviewed recently by Kempner (53), irradiation of complex, multicomponent enzyme systems does not cause disruption of complexes, but introduces breakages in the protein backbone along each chain of the complex. Thus, although activity is lost, the decay in activity accurately reflects the loss in functional mass.

Most surprising and initially puzzling were the findings that irradiation of both the liver and adipocyte systems prior to exposure to regulatory ligands (hormones, fluoride ion, guanine nucleotides) displayed functional target sizes of about 1500 kDa for the stimulatory processes

involving glucagon + GTP; an even larger functional size was exhibited by the inhibitory phase of the adipocyte adenosine-receptor-mediated process. Such large sizes did not fit with the estimated sizes of receptors, G-proteins, or adenylyl cyclase. When the systems were exposed first to activating ligands and then analyzed for their target sizes, dramatic reductions in functional mass were observed. For example, in the presence of glucagon and GTP, the functional size was reduced to about 350 kDa. In the presence of fluoride ion or Gpp(NH)p, the size was reduced to about 250 kDa. The size of adenylyl cyclase as measured with MgATP as substrate was about 120 kDa, now realized from the structure of cloned cyclases.

Disaggregation Theory of Hormone/GTP Action

Out of these findings arose the postulate that the hormone-sensitive cyclase system is composed of an oligomeric complex of receptors and G (or N) proteins which, upon interaction with hormone and GTP, disaggregate into monomers of the receptor-G complex (33).

Most importantly, target analysis led me to the conclusion that the primary signal emanating from the actions of hormones must be a protein; this protein had to consist, minimally, of a GTP-binding protein. Not knowing that G-proteins were heterotrimers, the estimated size of the monomer ranged from about 120 kDa [fluoride- or Gpp(NH)p-activation] to about 220 kDa after glucagon-treatment (correcting for the estimated mass of cyclase). The estimated values obtained after fluoride or Gpp(NH)p treatment were much larger than that of G_{α_s} (43–50 kDa). The larger value obtained after glucagon treatment I conjectured as the combination of the receptor complexed with a monomer of G_s . The monomer complex, considered to be the true “messenger” of hormone action, reacts with adenylyl cyclase resulting in either stimulation (by G_s) or inhibition (by G_i). This theory I termed the “Disaggregation Theory of Hormone Action” (33). Incorporated are the fundamental ideas that the structure of the receptor-G-protein complex is a multimer of these components, that adenylyl cyclase exists separately from the complex, and that a “monomeric” structure derived from the disaggregation is the messenger that communicates information from the hormone-bound receptor-G-protein complex to the effector or enzyme.

In this model, I had assumed that receptors and G-proteins existed in about equal amounts and were coupled stoichiometrically. Much later when accurate

methods became available for measuring the concentrations of receptors and G-proteins in cells, it became clear that in most cells, G-proteins are present in excess of receptors, possibly as much as 10:1. Given such information, clearly the model must be altered in that the largest portion of the mass of the glucagon-sensitive adenylyl cyclase (or the adenosine-sensitive, inhibitory system in adipocytes) must be attributed to that of G-proteins i.e., G-proteins are likely multimeric structures.

The disaggregation theory soon fell into disfavor because of the findings that heterotrimeric G-proteins treated with Gpp(NH)p or the later, more popular GTP γ S dissociated into free α -subunits and the $\beta\gamma$ complexes (54,55). From this arose the “dissociation” theory (Gilman, 1988). On my part, the disaggregation theory clearly needed biochemical evidence for the existence of multimeric forms of G-proteins. The odyssey in this direction began with two approaches: cross-linking experiments with synaptoneurosomes from rat brain and extraction of G-proteins with various detergents followed by sucrose-gradient analysis of the hydrodynamic properties of the extracted material.

Cross-linking Studies

Synaptoneurosomes were chosen for most of the studies because brain tissue contains the bulk of all known types of G-proteins. We were greatly aided in these studies by generous contributions from several colleagues (principally, Alan Spiegel at NIH) in the field who had prepared polyclonal antibodies against peptide sequences of the α and β subunits of several types of G-proteins (G_s , G_i , G_o , and G_q), including subspecies of these proteins.

We tested a variety of cross-linking agents for both their efficacy and selectivity of action at low concentrations. Phenylenedimaleimide proved the most satisfactory. In addition to all of the G-proteins tested, multimeric tubulin and F-actin were the only two types of membrane-associated proteins that were detectably cross-linked (56). After cross-linking in their membrane-environment, the G-proteins were extracted with sodium dodecyl sulfate and chromatographed on sieving columns that allow separation of proteins over a large range of sizes. In this manner it was found that both α - and β -subunits of G_s , G_i , G_o , and G_q were cross-linked to form structures comparable in size to cross-linked tubulin or actin. We concluded from these studies that G-proteins, most likely intact heterotrimers, are multimeric structures in association with the plasma membrane. Such evidence provided substantial credence to our basic arguments for the disaggregation theory.

Most importantly, it appeared that multimeric G-proteins are responsible for the large ground-state structures observed with target analysis.

Detergent Studies

The next stage necessitated some means of isolating the multimeric G-proteins, a process necessitating the use of detergents. Aware of the fact that detergents such as sodium cholate and Lubrol extracted intact heterotrimeric structures (57); i.e., monomers of the putative multimers, we considered the possibility that these detergents may disrupt the multimeric structure. Accordingly, we tested the sizes of G-protein structures extracted with a variety of detergents, using hydrodynamic properties on sucrose gradients as our assay. Of the seven tested, octyl glucoside (OG), Tween 20, and digitonin yielded structures behaving hydrodynamically larger than those given with sodium cholate or Lubrol, after correcting for the possible contributions of micellar forms of the detergents (58). OG extracted from liver membranes structures that were heterodisperse, about 10% sedimenting through sucrose gradients, the bulk remaining soluble in the detergent. When membranes were treated with cholera toxin in the presence of 32 P-NAD (the means of specifically labeling G_{α_s}), the majority of labeled material appeared in the insoluble fraction (59,60). When such labeled material in the membranes was subjected to the combined actions of glucagon and low concentrations of GTP γ S, a large portion of the insoluble material became soluble and appeared in a fraction similar to that of purified heterotrimeric G_s .

Based on the cross-linking and hydrodynamic studies, we deduced that G_s is likely multimeric in liver and synaptoneurosomes membranes, that only multimeric structures are altered by glucagon and low concentrations of GTP γ S in liver membranes, and that one of the primary results of their action is the disaggregation of multimers to monomers, as predicted in the disaggregation theory. In synaptoneurosomes, high concentrations of GTP γ S caused dissociation into free α and $\beta\gamma$ of heterotrimeric G-proteins dissolved in Lubrol or sodium cholate but not in digitonin (58). Hence, our suspicions were confirmed that the native structures of G-proteins are not preserved with detergents used for purifying heterotrimeric forms of G-proteins.

Extended Disaggregation Theory of Hormone Action

Target analysis provided the initial impetus for proposing the disaggregation theory. However, it has become clear that the the-

ory as originally presented has to be modified to account for the fact that G-proteins are the major component representing the large functional mass; i.e., G-proteins form multimeric structures. We had also established that there are marked differences between the regulation of G-proteins by the coupled receptors and the regulation of adenylyl cyclase by G-proteins (42,43). When the structures and regulatory properties of adenylyl cyclases became known (61), particularly the fact that these are transmembrane proteins that have a two-cassette structure (i.e., two distinct domains on a 12 membrane-spanning structure,) it became possible to construct a more coherent theory to explain the regulation of the cyclase system (62). Two regulatory cycles, one for regulation of multimer to monomer G-proteins, the other for regulation of cyclase by a monomeric G-protein (G_s) are illustrated in Figure 2.

The excursion of receptor along the multimeric G-protein chain is governed by the hormone-induced exchange of GTP and GDP; the GTP-occupied monomer at one end is released, allowing it either to interact with adenylyl cyclase or to return (after hydrolysis of GTP to GDP) to the other terminus of the multimer. In cycle B, the GTP-occupied monomer interacts

with the enzyme without necessarily inducing significant changes in enzyme activity. Activity is governed by magnesium-dependent hydrolysis of bound GTP to GDP + P_i . In this theory hydrolysis induces dissociation of α from $\beta\gamma$; the resultant separated subunits interact distinctively with the two cassettes or domains of adenylyl cyclase. Depending on the type of adenylyl cyclase associated with the associated G-protein, activity is governed solely by α_s , synergistically by the combination of α_s and $\beta\gamma$, or by inhibition of α_s -stimulation by $\beta\gamma$. Release of P_i from its binding site on α_s results in reassociation of α_s with $\beta\gamma$. The GDP-bound G_s then reassociates with the multimer to become part of the hormone-regulated cycle. It should be emphasized that both cycles occur in association with the surface membrane. The principal element that differs from other theories of hormone-regulated cyclase systems is that the concerted interactions of enzyme, Mg^{2+} and GTPase are responsible for separation of α_s from $\beta\gamma$. The extent and duration of enzyme stimulation are controlled by the independent actions of the separated subunits and the rate at which P_i is released following hydrolysis.

Most people in the field will argue that hydrolysis is not necessary for activation because nonhydrolyzable analogs of GTP

are fully capable of stimulating cyclase activity. However, my view is that allosteric regulation by Gpp(NH)p, a slow, hysteretic process, may involve stabilization of a magnesium-induced disassociation of G_s that normally exists transiently and which does not require any participation by adenylyl cyclase in the dissociative process. In this sense, the nonhydrolyzable analogs of GTP may have misguided many in the G-protein field into thinking that energy derived from the splitting of GTP is not involved in signal transduction. It should be noted in this extension of the disaggregation theory that both disaggregation of multimers and dissociation of monomers are separate but interrelated phenomena, both contributing to the overall dynamics of signal transduction.

G-Proteins Are Similar to Cytoskeletal Proteins

During these studies, my attention was drawn to the striking similarities in the properties of G-proteins with those of tubulin and actin, the major cytoskeletal elements in cells [which I have reviewed (63)]. For example, G-proteins, like actin and tubulin, are associated with the inner aspect of the surface membrane, adhering possibly both through intrinsic membrane proteins, such as receptors, and to membrane lipids. Of particular interest is the fact that all three types of multimeric proteins are subject to regulation by either GTP (G-proteins and tubulin) or ATP (actin) and their hydrolytic products (dinucleotides and P_i). Receptors regulate exchange of bound nucleotides (GDP with GTP) and act catalytically in the process. Similarly, the excursion of a single myosin molecule during muscle contraction along the chain of actin multimers is governed by the exchange of bound ADP with ATP and the hydrolysis of ATP to ADP and P_i . As stated previously, GTP turnover (production of GDP + P_i) is essential for the rapid and sustained actions of hormones; release of bound P_i is the crucial rate-limiting process in the overall dynamics of signaling. The same is true for myosin/actin interactions (64).

With these similarities in structure and regulation, G-proteins can be classified as part of the cytoskeletal matrix, with the primary functional difference that G-proteins serve as chemical signaling devices, whereas tubulin and actin serve as mechano-signaling devices. The release of monomers from multimers is the basis for chemical signaling by G-proteins. Dynamic changes in the disaggregation–aggregation cycle of actin and tubulin multimers are fundamental for regulating the interactions or movement between specialized components of cells. Based on evidence accumulated over the

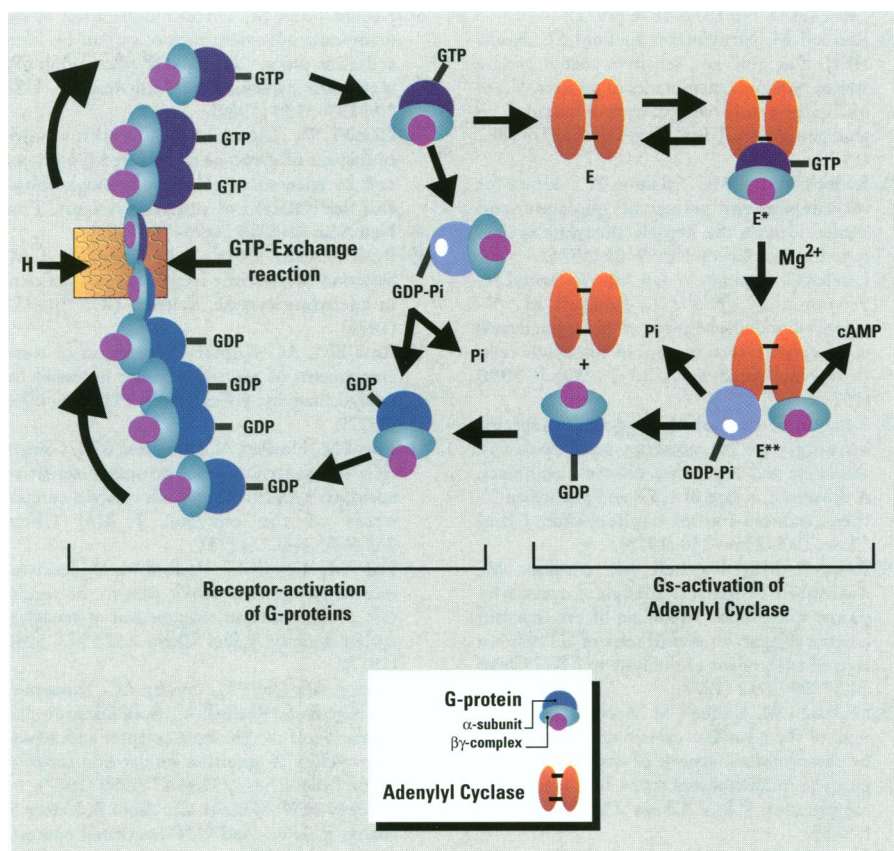


Figure 2. Regulation of the adenylyl cyclase system through receptor activation of G-proteins and the activation of adenylyl cyclase by G_s .

past decade (63), all three types of cytoskeletal proteins are connected in some manner to a variety of signaling systems that adhere to the cytoskeletal matrix, including heterotrimeric G-proteins, so-called small molecular weight G-proteins, protein kinases and phosphatases, and other proteins or systems that communicate between the surface membrane and the interior of cells. These components form weblike structures that possibly interact in a flickering manner in response to activation of membrane receptors, including those that are growth promoting.

Given the extraordinary complexity of signaling processes, as viewed at the biochemical level, clearly needed are new investigatory tools. Already promising are the microscopic imaging techniques with immunofluorescent molecules for specifically tagging and viewing structures in their living environment. I suspect that the reductionists with their prowess in molecular biology and X-ray crystallography and those of us attempting to view the living process at the cellular level will merge with our assemblages of ideas and experiences. When this larger, multiplex community of effort finally is consummated, a bright new era in scientific discovery will certainly emerge.

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